基础研究

Bioinformatics analysis of exosome proteome derived from hepatic carcinoma HepG2 cells

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Abstract **Objective**: The aim of this study was to analyze the exosome proteome derived from hepatic carcinoma HepG2 cells and normal hepatocytes HL-7702, and look for the key factors in carcinogenesis of hepatocellular carcinoma (HCC). **Methods**: HepG2 and HL-7702 cells were cultured, and exosome samples were obtained from the culture supernatant and verified. LTQ-Orbitrap Elite mass spectrometry was applied to analyze and identify the exosome proteome derived from the hepatic carcinoma cells and normal liver cells, which was for seeking hepatic carcinoma cells specifically expressed proteins. Furthermore, gene ontology (GO), protein-protein interaction (PPI) network, and pathway enrichment were constructed to analyze hepatic carcinoma cells specifically expressed proteins. **Results**: The exosomes of HL-7702 expressed 3, 366 proteins, and the exosomes of HepG2 expressed 2, 874 proteins, of which 1, 224 were expressed specifically in HepG2 exosomes. 102 target proteins were selected and going on bioinformatics analysis under the condition that the unique peptide was ≥1 and PSMs ≥10. GO analysis results showed that the target expression protein of HepG2 was concentrated on metabolism, proliferation, and localization adhesion function. 76 target proteins were chosen and formed a PPI network; combining with pathway analysis, ACTN1, FN1, RAC1 and GSN were found to be involved in important signaling pathways of HCC. The 26 target proteins which not in PPI network were analyzed pathway involved in, respectively. AKR1C2 and C5 were found to be involved in differently important signaling pathways of HCC. **Conclusion**: Exosomes proteomics provides the sources of specific protein derived from hepatic carcinoma cell; when it combines with bioinformatics analysis, they are able to reveal the important molecules that are related to carcinogenesis of HCC and provide new ideas for investigation of HCC biomarkers.

Keywords liver cancer; mass spectrometry; bioinformatics analysis

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Introduction

Exosome is composed of bilayer phospholipids molecules in the vesicles. It's distributed in the peripheral blood, urine, saliva, ascites and other body fluids[1]. Exosome carries the source cells of pathological and physiological markers of protein, mRNA, miRNAs, and some other signaling molecules; these particularly small molecules may be associated with organ-specific disease [2-4]. In 2007, Valadi et al. [5] found that cells could exchange genetic material by RNA in exosomes and participated in cell-cell communication. Exosomes that were secreted by liver cancer cells could carry the signal molecules from hepatocellular carcinoma cells, and the surface membrane structures of vesicles could block the exchange of molecules within the microenvironment of the body. Analyzing the proteins that are carried by the extracellular vesicles and finding the exosome-specific expressed proteins is possible to reveal the key molecules and the markers of HCC.

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Materials and Methods

Preparation and validation of exosomes

HepG2 cells and HL-7702 cells were cultured in a 5% CO_2 incubator at 37 ℃. The cells were cultured in the continuous adaptation serum-free medium. The supernatant of HepG2 and HL-7702 cells were collected and centrifuged by modified ultracentrifugation, and they were collected and transferred to eppendorf tubes. After resuspension, 20 μL of supernatant was placed on the sample-loaded copper mesh. The exosomes were identified by transmission electron microscopy (TEM). RIPA lysate was added to the precipitation, and 30 μL protein was used for SDS-PAGE. After the membrane was transferred, blocked, and eluted, adding ALIX, CD63, CD9 monoclonal antibody to react overnight at 4 ℃. Eluting it again and adding fluorescent secondary antibody to react for 1 h. Finally, the results of ODYSSEY double-color thermal infrared imaging system scan showed that precipitation contained exosomes marker protein.

LTQ-Orbitrap Elite mass spectrometry was used to obtain exosomes proteome

Protein samples were prepared with 5-fold acetone and quantified by Qubit Protein Assay Kit instructions, and its quality was measured by SDS-PAGE. The enzyme hydrolyzed protein sample was enzymatically digested, taking the same amount of mass and volume of the sample, using iTRAQ Quantitative Reagent, and separated by high-performance liquid chromatography (HPLC) on a C18 reversed phase column. The detached fraction was subjected to desalination processing and vacuum extraction, and then conducted by LTQ-Orbitrap mass spectrometry. According to the raw data of LTQ-Orbitrap Elite mass spectrometry, the high-confidence peptides (greater than 95%) were taken by searching and matching the human species in UniProt library using SEQUEST searcher of Proteome Discovery software. The search parameters were set as follows: Digestive enzymes trypsin, non-specific digestion, oxidation and carbamidomethyl C of methionine[6].

Analysis of exosome proteome derived from the hepatic carcinoma cells by gene ontology

The Vien map was used to screen the specifically expressed proteins in the exosomesderived from the hepatic carcinoma cells. Isomer-protein, although the same molecular formula, the molecular arrangement, physical and chemical properties were different, so in this study, isomers were treated as different proteins. The target protein was screened by the standard: specific peptides ≥1 and PSMs≥10. Uploading them to the online software DAVID (https://david.Ncifcrf.gov/tools.jsp), the unidentified accession was manually retrieved by GeneBank database on NCBI. Gene ontology was conducted by DAVID to analyze the molecular function, biological pathway, and the annotation of cellular components of target protein.

Construction of target protein PPI network

The name of normalized protein was uploaded to STRING 10.0 software (http://string-db.org/) to construct a PPI network consisting of the products that expressed by the introduced gene. The network interaction data of software STRING 10.0 was put into software CytoScape 3.2.1 for further visualize the whole network. At the same time, CentiScape was used to calculate the topological characteristics of the whole network and each node for selecting the key proteins, $P<0.05$ is considered to be significant difference.

Analysis of pathways of target proteins

The key proteins from the protein network were transferred to DAVID for KEGG pathway analysis, and the pathways of enrichment were analyzed. KEGG database was also used to analyze the pathways of non-interacting target proteins and screened the proteins that were involved in the pathways of HCC.

Results

Mass spectrometry analysis of the exosome proteome derived from hepatic carcinoma HepG2 cells and normal hepatocytes HL-7702

The peptides were found to be more than 95% con-

fidence, and 3,366 proteins were found in the exosomes of HL-7702 by mass spectrometry. 2,874 proteins were expressed in the exosomes of HepG2 including 1,224 specific expression in the exosomes of HepG2 cell line (Fig. 1). Further screening conditions were set to be specific peptide $\geqslant 1$ and PSMs $\geqslant 10$, which resulting in 102 target proteins derived from hepatic carcinoma HepG2 cells, included 10 pairs of structural isomeric proteins.

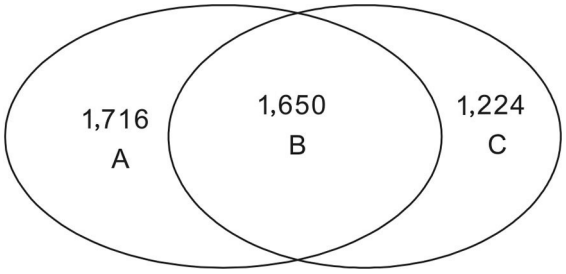

Fig. 1 Vien map of exosome proteome. A: HL-7702 cell line exo-specific expressed proteins; B: Both cells expressed exosome proteins; C: HepG2 cell line exo-specific expressed protein.

GO analysis of target proteins

102 target proteins wereimplemented by software DAVID. The results showed that, according to molecular functional annotation (MF), these target proteins were involved in protein binding (39.2%), catalytic activity (34.2%), molecular structure (16.5%), and transporter activity (6.7%). According to the classification of biological processes (BP), most of the target proteins were involved in cell processes (53.2%) and followed by metabolic process (39.2%), cellular component organization or biogenesis (29.1%), localization (20.3%); while 11.4% and 3.8% of the proteins were involved in the developmental process and bioadhesion, respectively. Following the instructions from cellular components (CC), 102 target proteins were mainly located in the cell part (27.8%), extracellular region (16.5%), organelles (16.5%), and macromolecular complex (11.4%).

Analysis of key proteins in PPI network

102 target proteins were uploaded to online software STRING 10.0 to construct thePPI network. Among them, 16 proteins were not associated with the other proteins or the main network, which suggested these nodes had no effect on the whole network. The interaction data of STRING gene expression products were imported into software Cytoscape, the topology of the whole network and each of the nodes was calculated by CentiScape. The key nodes were screened out. The network was composed of 76 nodes and 231 edges with a diameter of 7.00 and an average distance of 2.84. The maximum node degree value was 29.00, the minimum was 1.00, and the average was 6.08. The maximum node betweenness value was 1,286.41, the smallest was 0.00, and the average was 132.26. The larger the node degree, the more the protein interacted within the proteins. The larger the betweenness value, the greater the effect of this point on the regulation of the whole network. 16 nodes in the network whose nodes degree and nodes betweenness were simultaneously greater or equal to their mean (Table 1).

Table 1 The key proteins screened from PPI network

	accession	name	node degree	node betweeness
1	P02768	ALB	1,286.41	29.00
2	P63000-2	RAC1	1,246.01	21.00
3	P04406-2	GAPDH	1,175.02	27.00
4	P00734	F2	504.94	15.00
5	P00747	PLG	466.09	17.00
6	P68032	ACTC1	323.37	11.00
7	P12814-2	ACTN1	286.88	11.00
8	Q13813-3	SPTAN1	279.56	9.00
9	P13929-3	ENO3	274.39	8.00
10	P02751-14	FN1	213.10	13.00
11	P14543-2	NID1	211.23	7.00
12	P15311	EZR	203.27	11.00
13	Q00839-2	HNRNPR	192.78	7.00
14	P06748-2	NPM1	182.46	7.00
15	P53396-2	ACLY	182.37	11.00
16	P06396-2	GSN	137.06	11.00

Pathway enrichment analysis of key proteins

Using DAVID to analyze the key proteins, sixteen key proteins were found to be involved in six pathways, and two of them were found to be related to HCC: Focal adhesion and Viral carcinogenesis (Table 2). In these two pathways, there were four key proteins that involved in the pathogenesis of HCC: ACTN1, FN1, RAC1 and GSN, which were all related to HCC and possible to be important proteins in the carcinogenesis of the HCC.

Table 2 Enrichment of 16 key proteins pathways

pathway	count	protein	*P* value	relation with HCC
Regulation of actin cytoskeleton	6	ACTN1, F2, EZR, FN1, GSN, RAC1	<0.05	U
Leukocyte transendothelial migration	3	ACTN1, EZR, RAC1	<0.05	U
Proteoglycans in cancer	3	EZR, FN1, RAC1	<0.05	U
Biosynthesis of antibiotics	3	ACLY, ENO3, GAPDH	<0.05	U
Viral carcinogenesis	3	ACTN1, GSN, RAC1	<0.05	C
Focal adhesion	3	ACTN1, FN1, RAC1	<0.05	C[7-11]

U: uncorrelated; C: correlated.

Analysis of target proteins outside the PPI network

26 target proteins were not associated with the other target proteins or the main network. Removing 10 structural isomeric proteins, 16 proteins were analyzed by KEGG pathway database, respectively. Except three proteins AKR1C2, C5, and CXADR, the other proteins were not found involved in any of pathways (Table 3). AKR1C2 was involved in steroid hormone biosynthesis pathway, there were reports about the pathways that associated with HCC. C5 was involved in the complement and coagulation cascades, Staphylococcus aureus infection, and systemic lupus erythematosus, which were all associated with HCC.

Table 3 Target proteins outside PPI network

accession	name	unique peptide	PSMs	pathway	relation with HCC
B4DK69	AKR1C2	5	12	H	H[12]
M0R116	ATP1A3	1	48	N	N
P01031	C5	6	11	H	H[13, 14]
H0Y5N9	COL12A1	1	139	N	N
P78310-7	CXADR	9	14	H	H
P01876	IGHA1	6	11	N	N
P01857	IGHG1	5	33	N	N
P01859	IGHG2	2	19	N	N
P01871	IGHM	8	21	N	N
P01834	IGKC	4	18	H	H
B9A064	IGLL5	2	10	N	N
P04259	KRT6B	1	31	N	N
P48668	KRT6C	1	26	N	N
Q32Q12	NME1-NME2	2	17	N	N
P20742	PZP	2	35	H	H
A6NIZ1	RP1BL	1	12	N	N

H: have pathway or relation with HCC; N: no pathway or no relation with HCC.

Discussion

The etiology of HCC is complicated and potentially linked to HBV, HCV, aflatoxin, liver cirrhosis and some other causes that induce liver diseases [15]. Although its molecular mechanism is unclear, the molecular mechanism of HCC is highly essential for HCC diagnosis and treatment. Mass spectrometry is one of the most powerful tools for the identification of pure substances. LTQ-Orbitrap Elite mass spectrometry is a combination of high efficiency, sensitivity, high selectivity, and has a great advantage in the protein identification. In this study, the exosomes of HepG2 and HL-7702 cells were studied by LTQ-Orbitrap Elite mass spectrometry. The results showed that there were 2, 874 proteins in HepG2 exosomes, among them 1, 224 proteins specifically derived from the hepatic carcinoma HepG2 cells. After further screening by the condition that the unique peptide was ≥1 and PSMs ≥10, 102 target proteins were selected for subsequent analysis, included 10 pairs of isomer proteins.

The molecular functional annotation indicated that these target proteins were involved in protein binding, catalytic activity, molecular structure-activity, and transporting activity. The biological pathway classification indicated that these proteins were participated in cellular processes, metabolism, biological tissue components, and localization; the cell division showed that these proteins were mainly expressed in cells protein, the extracellular domain,

and macromolecule complex. Comparing with exosome proteins derived from normal hepatocytes, the proteins that expressed in exosomes of HepG2 cells were concentrated in metabolic, proliferative, and localized adhesion. Consistent with their characteristics, they were active in tumor cell metabolism, rapid proliferation, loss of differentiation ability, localization and adhesion capacity enhancement, and so on.

In the protein regulatory network, the node proteins played an important role in the stability of the network. In a PPI network, the importance of proteins in the network was evaluated by the node degree and node betweenness. The larger the two values, the greater the role and influence of this node protein in the whole network. According to the node degree and node betweenness mean value, 16 node proteins were selected as key proteins for further enrichment pathway analysis, and two pathways were found to be associated with HCC. The results showed that four proteins ACTN1, FN1, RAC1 and GSN involved in might be the crucial proteins in the carcinogenesis of HCC. PPI network analysis revealed 26 target proteins were not associated with the other proteins or the main network. KEGG pathway database showed that protein AKR1C2 and C5 were involved in the pathogenesis of HCC.

ACTN1 is an anchored protein of many intracellular structures that is coupled to actin and plays a crucial role in the cellular motility and migration. Moreover, it's connected to the intercellular adhesive molecule interaction and involves in the inflammatory response. In recent years, α-actinin has implicated in the development and progression of various types of tumors, and it is involved in the movement, metastasis, and invasion of many kinds of tumors [16-21]. α-actinin gene was highly expressed in hepatocarcinoma cells with the malignant phenotype [22]. FN1 is a fibronectin that binds to the cellular surface and related to various complexes including collagen, fibrin, heparin, DNA, actin and so on. It is involved in cell adhesion, cell movement, wound healing, conditioning, and the maintenance of cell morphology. The reports about FN1 and HCC have demonstrated a certain relationship between these two [23-24]. RAC1 is a member of RAC family. It regulates the activities of different cells including cell growth, cytoskeleton remodeling, and protein kinase activation. As a result, RAC1 is an important signal transduction molecule in the cells; it can convert between GTP and GDP. After GTPase binds to GTP, the conformation will change and bind to the downstream substrate to transduce the signal. Yang et al. [25] evaluated 242 cases of HCC and adjacent tissues found that RAC1 was higher in cancer tissues on higher clinical stage (Ⅲ-Ⅳ) and higher levels of alpha-fetoprotein. Besides, up-regulation of RAC1 is linked to vascular invasion and hepatic metastasis, and tissue differentiation. Postoperative survival analysis showed that increasing RAC1 indicated the poor prognosis of HCC. RAC1 is associated with the migration and invasion of breast cancer cells [26]. Also, it is used as the potential therapeutic target for anti-chemotherapy of head and neck squamous cell carcinoma [26]. GSN is also involved in the invasion and metastasis of hepatic carcinoma cells [14, 27]. By using bioinformatics analysis, we found that RAC1, ITGA2, FN1, and ACTN1 are either directly or indirectly related to HCC and other types of tumors mainly in tumor motility, metastasis, metastasis, and tumorigenesis. This finding can be used as an entry point of the molecular mechanism of exosomes research. Target proteins outside the PPI network are also under concern. Despite lacking evidence, there are reports about AKR1C2 and C5 are participated in the infiltration and metastasis of HCC[12-14].

Comprehensively, exosomes proteomes is capable of providing the sources of specific proteins derived from hepatic carcinoma cells. Combining with bioinformatics analysis, a new way of investigating HCC-relevant important molecules and biomarkers can be found.

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Conflict of interest The authors declare there are no competing interests.

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